

Demethylation of DNA by purified chick embryo 5-methylcytosine-DNA glycosylase requires both protein and RNA

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ABSTRACT

We have previously purified and characterized a 5-methylcytosine (5-MeC)-DNA glycosylase from 12 day old chick embryos [Jost, J.P. *et al.* (1995) *J. Biol. Chem.* 270, 9734–9739]. The activity of the purified enzyme is abolished upon treatment with proteinase K and ribonuclease A. RNA copurifies with 5-MeC-DNA glycosylase activity throughout all chromatographic steps and preparative gel electrophoresis. RNA with a length of ~300–500 nucleotides was isolated from the gel purified enzyme. Upon extensive treatment with proteinase K, the gel eluted and labeled RNA did not show any significant change in molecular mass. The purified RNA incubated alone or in the presence of Mg^{2+} and deoxyribonucleotide phosphates had no 5-MeC-DNA glycosylase or demethylating activities. However, activity of 5-MeC-DNA glycosylase could be restored when the purified RNA was incubated with the inactive protein, free of RNA.

INTRODUCTION

There is strong evidence to suggest that the pattern of DNA methylation is vital for the normal development of vertebrates (1–5). It is believed that at least three different components are needed for the establishment of a specific methylation pattern: DNA methyltransferases, the demethylation system of DNA (passive and/or active) and the determination factors (*cis* and *trans*). DNA methyltransferases and the demethylation system are CpG or CpXpG specific but not strictly sequence specific whereas the determination factors should be sequence and developmental stage specific. Active DNA demethylation could be carried out by a 5-methylcytosine (5-MeC)-DNA glycosylase (6) or by nucleotide excision (7). In the first case there is evidence that a 5-MeC-DNA glycosylase is present in developing chick and mouse embryos (6,8) and in differentiating G8 mouse myoblasts (9). The enzyme is at least 10 times more active on hemimethylated rather than symmetrically methylated DNA (6). A similar activity has been detected in HeLa cells where the enzyme was shown to remove 5-MeC from symmetrically

methylated DNA (10), however, this enzyme may be related to a nuclease (11). More recently it has been shown that in whole cell lysates prepared from L8 mouse myoblasts and from F9 teratocarcinoma cells, DNA could be demethylated by RNA through a mechanism of nucleotide excision repair (7). In our present case we show evidence that RNA copurifies with 5-MeC-DNA glycosylase and that both RNA and protein are necessary for the activity of the enzyme.

MATERIALS AND METHODS

Purification of chick embryo 5-MeC-DNA glycosylase

Purification of crude nuclear extracts from 12 day old chick embryos, chromatography on heparin-sepharose, DEAE-sepharose, carboxymethyl (CM)-sepharose and affinity chromatography on DNA-Dynabeads were carried out as previously described (6). Following chromatography on CM-sepharose we included an additional purification step on butyl-sepharose. The ammonium sulfate sediment from the active fraction eluted from CM-sepharose was slowly dissolved in 1.2 M $(NH_4)_2SO_4$ containing 20 mM HEPES pH 7.5, 10 mM EDTA, 100 mM KCl, 2 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonylfluoride (PMSF). Non-solubilized protein was sedimented by centrifugation and discarded. The supernatant fraction, containing 10–15 mg protein was loaded onto a 10 ml column of butyl-sepharose 4B equilibrated with 1.7 M $(NH_4)_2SO_4$, 20 mM HEPES pH 7.5 and 1 mM DTT. The column was step-eluted with buffers containing 1.7, 1.5 and 1.3 M down to zero M $(NH_4)_2SO_4$. The main peak of activity eluting with 0.85 M $(NH_4)_2SO_4$, was precipitated with 0.47 g/ml of solid $(NH_4)_2SO_4$. The butyl-sepharose chromatography was followed by affinity chromatography on DNA-Dynabeads and preparative SDS-polyacrylamide gel electrophoresis exactly as described previously (6). The enzyme purified from the affinity column was precipitated with acetone. The 1 M NaCl eluate containing the active enzyme was diluted with water to 0.15 M NaCl and 4 vol acetone was added. Precipitation was carried out in silicone treated Eppendorf tubes and the precipitate was kept at $-80^\circ C$ until further use. Similarly the enzyme eluted from preparative gels with 0.15 M NaCl, 10 mM HEPES pH 7.5, 1 mM EDTA, 0.5% SDS, 1 mM DTT and 50 μg bovine serum albumin (BSA) (RNase and DNase free) was filtered

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through a 0.45 μ Millipore Ultrafree filter and precipitated as indicated above, where indicated BSA was replaced by 100 μ g dextran-T 70 as carrier. The enzyme was recovered from the acetone precipitate by centrifugation for 20 min at 12 000 r.p.m. in an HB-4 Sorval rotor at 2°C. The sediments were washed three times with cold acetone (acetone:water, 9:1 v/v) by vigorous vortexing and centrifugation at 17 000 r.p.m. for 5 min in an SS-34 Sorval rotor.

Purification of other proteins

The translation initiation factor eIF2 γ was purified from 12 day old chick embryos ribosome as described by Stringer *et al.* (12). The crude 0.4 M KCl ribosome extract was fractionated by (NH₄)₂SO₄, dialyzed and chromatographed on a DEAE-sepharose column (12). Mismatch-specific thymine-DNA glycosylase was purified from HeLa cells as described by Neddermann and Jiricny (13).

Enzyme assay

The assays for 5-MeC-DNA glycosylase and G/T mismatch-DNA glycosylase were carried out as described previously (6). The hemimethylated, end-labeled oligonucleotide (only the upper strand is shown) 5'-GGTATTCCTGGTCAGCGTGACm-CGGAGCTGAAAGAACACATTGATCCCGTG-3' was used as the standard substrate throughout all experiments. The reaction product was heat or alkaline denatured and analyzed on a 20% polyacrylamide-urea sequencing gel.

Peptide sequencing

The preparative SDS/10% PAGE was followed by electroblotting of the protein onto poly (vinylidene difluoride) membranes (Immobilon from Millipore). Tryptic digestion of the bands containing 5-MeC-DNA glycosylase activity was carried out according to Bauw *et al.* (14), essentially as described by Mayer *et al.* (15). Additional washing of the membranes with 0.8% Triton was included prior to blocking with poly(vinylpyrrolidone). The peptides were purified by reverse phase HPLC and sequenced as described (16).

RNA purification and radioactive labeling

The purified enzyme fraction was dialyzed against 0.15 M NaCl, 20 mM HEPES pH 7.5, 5 mM EDTA. SDS was added to a final concentration of 0.5% and the protein was digested for 1 h in the presence of 200 μ g/ml proteinase K. Samples were extracted four times with phenol and chloroform and the RNA was ethanol precipitated. Upon sedimentation the RNA was dissolved in 0.2 M NaCl and reprecipitated twice with ethanol. RNA dissolved in sterile water was stored for short periods of time at -80°C or for longer times in liquid nitrogen. RNA was labeled in a total volume of 10 μ l containing 30 μ Ci [γ -³²P]ATP, 5 U polynucleotide kinase, 5 mM DTT and 50 U ribonuclease inhibitor in 1 \times polynucleotide kinase buffer. Upon 30 min incubation at 37°C the reaction mixture was mixed with an equal volume of 2 \times loading buffer and fractionated on a 10% SDS-polyacrylamide gel.

Chemicals and enzymes

Benzamidine was purchased from Fluka AG (Buchs/SG, Switzerland). PMSF, Pefabloc, proteinase K, ribonuclease A (DNase free) and the inhibitor of ribonuclease were obtained from Boehringer

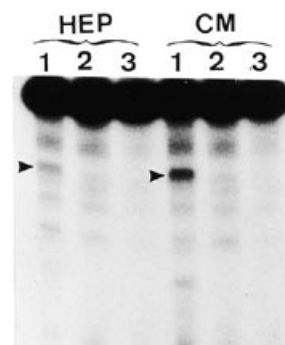


Figure 1. Effect of proteinase K on the activity of 5-MeC-DNA glycosylase. The reaction product was analyzed on a 20% polyacrylamide-urea DNA sequencing gel. Active fractions from heparin-sepharose (10 μ g protein, HEP) and CM-sepharose (1 μ g protein, CM) were tested. Lane 1 shows the positive control, first preincubated for 20 min at 37°C without substrate and then incubated further with the labeled DNA substrate for 1 h at 37°C. In lane 2, the enzyme fraction was pretreated with 100 μ g/ml of proteinase K for 20 min at 37°C. Labeled DNA substrate was then added and the incubation continued for 1 h at 37°C. Lane 3 is the negative control with DNA substrate incubated with BSA. The arrowheads show the position of the correct cleavage product.

Mannheim. Polynucleotide kinase and restriction enzymes were obtained from Biofinex (Praroman, CH-1724, Switzerland). Heparin-sepharose CL-6B, DEAE-sepharose, CM-sepharose fast flow and butyl-sepharose were obtained from Pharmacia Biotech Inc. Dynabeads-Streptavidin were purchased from Milan Analytica AG (CH-1634, LaRoche, Switzerland). Collodion dialysis bags were from Sartorius AG (D-3400, Göttingen, Germany). [α -³²P]dATP and [γ -³²P]ATP triethylammonium (3000 Ci/mmol) were purchased from Amersham.

RESULTS

Purified 5-MeC-DNA glycosylase is sensitive to proteinase K and ribonuclease A

Recently it has been shown that the demethylation of DNA is dependent only on RNA and that an extensive proteinase K treatment of crude cell lysates from mouse myoblasts did not alter the enzymatic removal of 5-methylcytidine from a methylated DNA substrate (7). In sharp contrast the results presented in Figure 1 show that a treatment of the purified 5-MeC-DNA glycosylase (fraction eluted from heparin-sepharose and from CM-sepharose) with 100 μ g/ml proteinase K abolishes the activity of the enzyme completely whereas the non-specific cleavage sites on the substrate shown above and below the arrow were unaffected. Similarly, a pretreatment of the enzyme preparation with 10 μ g/ml ribonuclease A (DNase free) for 20 min at 37°C also abolishes the activity of 5-MeC-DNA glycosylase purified on heparin-sepharose or butyl-sepharose columns (Fig. 2A and B). It could be argued that the effect of ribonuclease A is due to a protease contamination. This possibility was investigated by treating 5 U of the restriction enzyme *MspI* with increasing large concentrations of ribonuclease A prior to the incubation with the labeled DNA substrate. Figure 2C shows clearly that a preincubation of *MspI* for 20 min at 37°C with up to 1.5 mg/ml ribonuclease A did not abolish or alter the enzyme activity (Fig. 2C, lane 4). Furthermore, addition of up to 4 mM of 4-(2-aminoethyl)-benzenesulfonyl

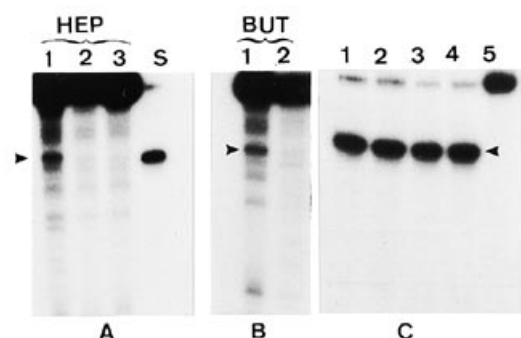


Figure 2. Effect of ribonuclease A on the activity of 5-MeC-DNA glycosylase. The reaction product was analyzed as for the experiments shown in Figure 1. The enzyme fractions eluted from (A) heparin-sepharose (40 μ g protein) and (B) butyl-sepharose (1 μ g protein) were tested. Lane 1 (A and B) is the positive control preincubated for 20 min at 37°C without the DNA substrate and then incubated with the labeled DNA substrate for 1 h at 37°C. In lane 2 (A and B) the enzyme fraction was first pretreated with 10 μ g/ml of ribonuclease A for 20 min at 37°C. Labeled DNA substrate was then added and the incubation was continued for 1 h at 37°C. Lane 3 (A) is the negative control of the DNA substrate incubated with BSA. The arrowhead shows the position of the correct cleavage product. (C) A control experiment testing the presence of protease in the ribonuclease A preparation. The restriction enzyme *MspI* (5 U) was incubated for 20 min at 37°C with 0 (lane 1), 0.5 (lane 2), 1 (lane 3) and 1.5 mg/ml (lane 4) of ribonuclease A. Incubation was then continued for 1 h at 37°C in the presence of labeled DNA substrate. Lane 5 is the negative control incubated for 1 h at 37°C in the presence of 50 μ g BSA. Lane S (A) is a size standard provided by DNA substrate digested with *MspI*.

fluoride (Pefabloc) to all incubation mixtures of Figure 2 did not change the results. This strongly indicates that the decrease in 5-MeC-DNA glycosylase activity observed upon ribonuclease treatment may be due to the destruction of an enzymatically active RNA present in the 5-MeC-DNA glycosylase preparation.

It has been previously reported (6) that the gel purified 5-MeC-DNA glycosylase also had a G/T mismatch-DNA glycosylase activity (see also Fig. 4). As shown in Figure 3, the activity of the G/T mismatch-DNA glycosylase present in the preparation of 5-MeC-DNA glycosylase (fraction post CM-sepharose) is also sensitive to proteinase K (Fig. 3A) and to ribonuclease A (Fig. 3B).

RNA co-purifies and co-migrates with highly purified 5-MeC-DNA glycosylase activity

Since there was evidence that RNA might be essential for the activity of 5-MeC-DNA glycosylase it was reasonable to investigate whether the gel purified enzyme also contained RNA. Figure 4 shows that the two protein bands b and c eluted from the preparative gel (panel A) when denatured and slowly renatured regained activity for both 5-MeC-DNA glycosylase and G/T mismatch DNA glycosylase (panels B and C). All other fractions eluted from the gel were inactive. The active enzyme eluted from bands b and c was pooled and acetone precipitated. RNA present in the precipitate was labeled as described in Materials and Methods. Labeled RNA was then separated on a 10% polyacrylamide-SDS gel typically used for the proteins. Lane 1 of Figure 5A shows that the labeled material migrated exactly at the position of the active enzyme (mw between 50 and 53 kDa) and an additional smaller band is visible at 44 kDa. Evidence that it is RNA is shown in lane 2 of Figure 5A where the labeled

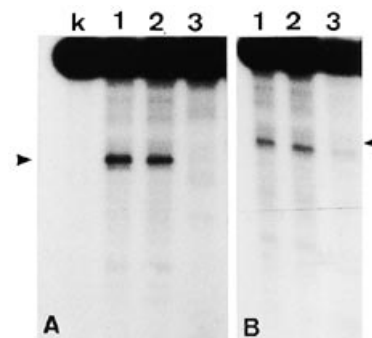


Figure 3. Effect of proteinase K and ribonuclease A on the activity of G/T mismatch-DNA glycosylase copurified with 5-MeC-DNA glycosylase (fraction post CM-sepharose). (A) Incubation with proteinase K (lane 3). The same conditions of incubation as for Figure 1 were used. (B) Incubation with ribonuclease A (lane 3). The incubation conditions were as for Figure 2. Lanes 1 and 2 (A and B) are the positive controls not preincubated (lane 1) and preincubated (lane 2) at 37°C for 20 min. k is the DNA substrate incubated in the presence of BSA. The reaction product was separated on a 20% denaturing polyacrylamide gel. The arrowheads indicate the correct position of the cleavage product.

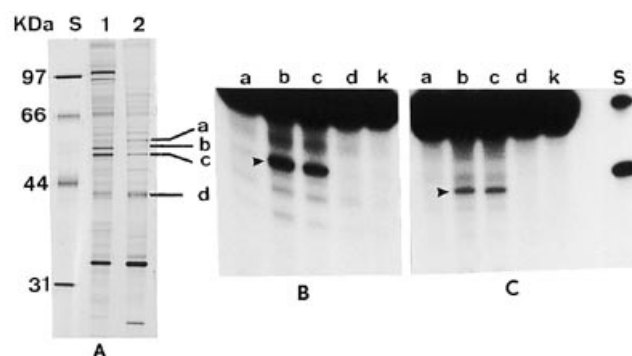


Figure 4. (A) Silver staining of the proteins separated on a 10% polyacrylamide-SDS gel. Lane S shows the protein size standards. Lane 1, 2 μ g of proteins not retained on the DNA-Dynabeads column (this fraction had no enzyme activity). Lane 2, 2 μ g of the protein retained on the affinity column (this fraction only had high specific activity of the enzyme). a, b, c and d represent the bands which were eluted from the gel and tested for 5-MeC-DNA glycosylase (B) and G/T mismatch-DNA glycosylase (C). The arrowheads show the correct position of the cleavage product. k is the control DNA substrate incubated with BSA.

preparation was hydrolyzed for 30 min at 56°C in 0.1 M NaOH. Lane 3 of the same figure shows that following an extensive proteinase K treatment there was no significant shift in the migration of the labeled RNA. A control incubated with [γ - 32 P]ATP but in the absence of polynucleotide kinase gave no trace of labeling (Fig. 5A, lane 4) indicating that the enzyme eluted from the gel had no endogenous kinase activity. When the labeled RNA, migrating at the position of the 5-MeC-DNA glycosylase, was extracted, denatured and rerun on a denaturing polyacrylamide gel, an approximate length of 300–500 nt was obtained (Fig. 5B, lane 1). Some product of degradation of the labeled RNA was also seen below the main bands. Further tests carried out with the nucleic acids extracted from the 5-MeC-DNA

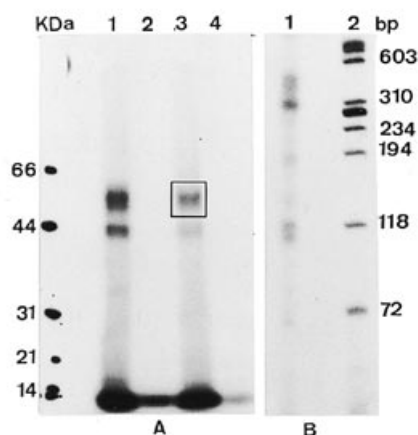


Figure 5. Labeling of the RNA from the pooled fractions b and c eluted from the SDS-PAGE shown in Figure 3. **(A)** The labeled RNA was separated on a 10% polyacrylamide-SDS gel as for the separation of the proteins. KDa are the protein size standards. Lane 1 shows the product of the polynucleotide kinase reaction. Lane 2 is the same as lane 1, except that the product of reaction was hydrolyzed with 0.1 M NaOH for 30 min at 56°C. Lane 3 is the labeled product subjected to proteinase K treatment (2 mg/ml) for 30 min at 37°C. Lane 4 is the labeling reaction carried out in the absence of polynucleotide kinase. **(B)** The analysis of the RNA extracted from the boxed area of (A) on a 6% polyacrylamide-urea denaturing gel (lane 1). Lane 2 shows the polynucleotide size standards in bp.

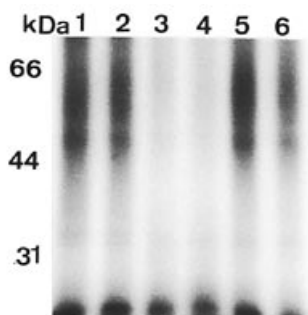


Figure 6. Sensitivity of the labeled RNA to ribonucleases and deoxyribonuclease I. RNA present in the 5-MeC-DNA glycosylase eluted from the CM-sepharose column was labeled as outlined in Materials and Methods. Labeled RNA was separated on a 10% SDS-polyacrylamide gel as in Figure 5. Lanes 1, 3 and 5, RNA not denatured; lanes 2, 4 and 6, RNA denatured for 2 min at 95°C. Lanes 1 and 2 are the positive controls not treated with enzymes. Lanes 3 and 4, RNA treated for 1 h at 37°C with a mixture of ribonuclease A (10 µg/ml) and ribonuclease I (200 U/ml); lanes 5 and 6, RNA treated for 1 h at 37°C with deoxyribonuclease I (200 U/ml).

glycosylase eluted from CM-sepharose indicated that it is sensitive to ribonucleases (Fig. 6, lanes 3 and 4) but not to deoxyribonuclease I (Fig. 6, lanes 5 and 6).

Purified RNA from 5-MeC-DNA glycosylase preparation alone has no catalytic activity

In the experiment presented in Figures 1 and 2 it is evident that the presence of both protein and RNA are absolutely required for the full activity of the 5-MeC-DNA glycosylase. However, in a different system such as ribonuclease P also requiring protein and

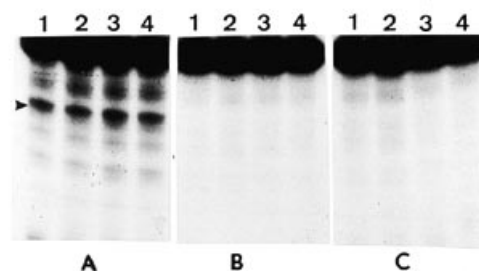


Figure 7. Activity of the purified RNA in the presence or absence of 5-MeC-DNA glycosylase. The panels show the product of reaction of 5-MeC-DNA glycosylase separated on a 20% polyacrylamide-urea sequencing gel. The arrowhead shows the correct position of the cleavage product. **(A)** The effect of increasing concentrations of purified RNA (RNA was purified from the active fraction eluted from heparin-sepharose) on the enzyme eluted from the CM-sepharose (1 µg protein). Lane 1, no RNA; lanes 2–4, 1, 5 and 10 µg RNA, respectively. **(B)** The activity of increasing concentration of the same purified RNA in the absence of purified 5-MeC-DNA glycosylase. The enzyme was replaced by 50 µg BSA. Lanes 1–4, 0, 1, 5 and 10 µg RNA, respectively. **(C)** The effect of increasing concentration of MgSO_4 on the activity of RNA in the absence of the purified enzyme. Purified RNA (5 µg) was incubated with 0, 10, 30 and 60 mM MgSO_4 (lanes 1–4). All incubations were carried out for 2 h at 37°C.

RNA it was shown that RNA alone could regain full catalytic activity under certain conditions of high Mg^{2+} concentrations (17). In Figure 7A, addition of more purified RNA to the already active enzyme did not result in any modification of its activity. When under the same incubation conditions, the same increasing concentrations of RNA alone were incubated in the absence of protein (panel B), no trace of 5-MeC-DNA glycosylase could be observed. A treatment of the reaction products with 0.1 M NaOH for 30 min at 90°C did not result in any specific cleavage of the labeled DNA template indicating that no removal of 5-MeC, in the absence of phosphodiester bond cleavage had occurred (results not shown). Inclusion in the reaction of 10, 30 and 60 mM MgSO_4 (panel C) did not result in DNA cleavage in the presence of RNA alone and further incubation in the presence of all four deoxyribonucleotide phosphates had no effect on DNA demethylation (data not shown).

Reconstitution experiments with the protein free of RNA and the RNA free of protein could restore the 5-MeC-DNA glycosylase activity

From the above experiments it is obvious that both protein and RNA are required for the full activity of 5-MeC-DNA glycosylase. Therefore, a combination of the purified RNA with the inactive enzyme free of RNA should restore 5-MeC-DNA glycosylase activity. Figure 8, lane 1 shows a positive control of the active enzyme eluted from a CM-sepharose column. A ribonuclease A pretreatment of the same enzyme preparation results in the loss of 5-MeC-DNA glycosylase activity (lane 2). For details of the experiment see Figure 8 legend. The enzyme pretreated with ribonuclease A could not be restored by the addition of tRNA (Fig. 8, lane 3). The same results were obtained by the addition of the same amount of crude cellular RNA consisting mostly of rRNA (data not shown). The activity of the enzyme pretreated with ribonuclease A could be restored by the addition of the purified RNA (RNA purified from an active enzyme preparation) (Fig. 8, lanes 4 and 5). Lane 6 is a negative

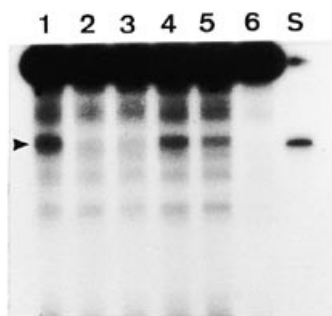


Figure 8. Reconstitution experiment with RNA (free of protein) and the protein fraction (free of RNA). The reaction product of 5-MeC-DNA glycosylase reaction was separated on a 20% polyacrylamide-urea gel. The arrowhead shows the correct position of the cleavage product. Lane 1 shows a positive control with the purified active enzyme (CM-sepharose fraction, 1 μ g protein) preincubated at 37°C for 20 min and then incubated further at 37°C for 1 h with the labeled DNA substrate. In lanes 2–5 the enzyme was pretreated for 20 min with 0.5 μ g ribonuclease A. Upon addition of 5 mM DTT (final concentration) and 200 U ribonuclease inhibitor, the reaction was continued by adding the labeled DNA substrate. Lane 3 received 5 μ g tRNA in addition to the labeled substrate and lanes 4 and 5 received 5 and 10 μ g of purified RNA, respectively (the same RNA preparation that was tested in Fig. 5). Incubation was continued for 1 h at 37°C and samples were processed for analysis on DNA sequencing gels. Lane 6 is a negative control consisting of DNA treated with ribonuclease A and incubated with 50 μ g BSA.

control of the substrate incubated with BSA. Further reconstitution tests carried out with the same preparation of RNA, but with proteins smaller than 52 kDa (10–40 kDa) eluted from the 10% SDS-PAGE, did not yield any trace of 5-MeC-DNA glycosylase activity (results not shown).

DISCUSSION

The enzymatic activity of RNA either alone or in combination with proteins has been well documented. For example, the group I ribozymes and ribonuclease P are able to cleave single stranded DNA *in vitro* (19–21) and the group II ribozymes can also ligate DNA to RNA (22). Group II intron RNA can associate with a DNA endonuclease and cleave one strand of the DNA duplex while the protein associated with the DNA cleaves the other DNA strand in a site-specific manner (23). In the case of ribonuclease P the RNA alone catalyzes the reaction only in the presence of very high concentrations of Mg^{2+} (17) while in the presence of low concentrations of Mg^{2+} it requires the presence of a protein. Other enzymes such as mitochondrial DNA primase (24) and telomere terminal transferase (25) are also associated with RNA.

The ‘demethylating’ enzyme that we previously characterized as 5-MeC-DNA glycosylase (6) also requires RNA and protein for its catalytic activity. That RNA could be copurified with the enzyme activity through several chromatographic steps including gel electrophoresis (enrichment of enzyme activity is up to 50 000-fold) is an indication that the interaction between the RNA and the protein must be very strong. Even the presence of 1.7 M $(NH_4)_2SO_4$ in the loading buffer of the butyl-sepharose column apparently did not dissociate the complex. However, the association of the RNA with the protein is most likely not covalent since an extensive proteolytic digestion of the complex of labeled RNA–protein with proteinase K did not result in a

significant change in the electrophoretic mobility of the RNA (see Fig. 5). However, within the limits of error the detection of any smaller protein (mw 5 kDa) or peptide covalently linked to the RNA may not be possible. One could argue that the protein moiety of the enzyme is proteinase K resistant. This is unlikely to be the case since the enzyme activity is very sensitive to proteinase K. Since throughout the purification of 5-MeC-DNA glycosylase no special precaution was taken to protect RNA against nuclease degradation, it is quite possible that our highly purified enzyme is associated with only the ‘core’ RNA of a larger RNA molecule. Therefore, it is not yet possible to exclude the existence of a larger RNA having additional catalytic functions. Once the sequence of the ‘core’ RNA associated with the 5-MeC-DNA glycosylase is known, it will be of paramount importance to look in our original crude nuclear extracts for the presence of a larger RNA precursor. Such a putative larger RNA molecule should be tested then for other catalytic activities related to DNA demethylation. While using cell lysates, Weiss *et al.* (7) have identified the presence of RNA capable of replacing the methylated cytidine in the DNA by an unmethylated cytidine. However there are some major differences between their system and ours. For example they showed that the product of the reaction was insensitive to the cleavage with NaOH at the site of demethylation, suggesting the absence of abasic sugar which normally occurs during the glycosylase reaction. In this specific case it is possible that any abasic sugar is immediately cleaved by enzymes present in the crude cell lysate. Their results taken together also suggest that demethylation of DNA is taking place through a process of nucleotide replacement rather than the action of a 5-MeC-DNA glycosylase. Moreover another major difference between their results and ours is the relative insensitivity of their system to proteinase K. Any serious comparison of the two systems will have to await the cloning of the molecules responsible for the demethylation reaction.

The protein nature of 5-MeC-DNA glycosylase has remained very elusive so far. In spite of considerable efforts invested in the purification of the enzyme, no relevant sequence has yet been found. This is mainly due to the extremely low concentration of the enzyme present in the developing embryos and the chromatographic and electrophoretic behavior of the enzyme. For example, the activity eluted from preparative gels never gave a single sharp band and the fractions with the highest specific activity were always present on the gel as a diffuse band (Fig. 4A, band b). The sharp band c of Figure 4A has been further characterized by peptide sequencing. Nine oligopeptides had 100% identity with the translation initiation factor eIF2 γ . We thought that this protein could either be part of the enzyme or it could be a major contaminant of 5-MeC-DNA glycosylase. The first possibility was tested by incubating increasing concentrations of the purified eIF2 γ alone or with 5 μ g of the purified RNA (same RNA preparation used for Fig. 7 experiments). The results clearly showed that eIF2 γ is not the protein moiety of 5-MeC-DNA glycosylase (data not shown). Comparison of the results of Figures 1, 2 and 3 shows that the two enzymes activities of 5-MeC-DNA glycosylase and G/T mismatch-DNA glycosylase are copurifying and comigrating on the same position on SDS–polyacrylamide gels (see ref. 6 and Fig. 4). Both enzyme activities are sensitive to ribonuclease and proteinase K. Neddermann *et al.* (13) have isolated from HeLa cells a G/T mismatch-DNA glycosylase which has no demethylating activity. In our laboratory, all attempts to reconstitute 5-MeC-DNA glycosylase

by combining the purified RNA from 5-MeC-DNA glycosylase and the G/T mismatch-DNA glycosylase purified from HeLa cells were negative, suggesting that the two enzymes isolated from chicken embryos and from HeLa cells are different (results not shown).

Obviously a different approach to purify the protein moiety of the enzyme is needed and the use of affinity resins containing the RNA may be one way to solve this problem.

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